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Analyses of Phosphorylation Events in the Rubella Virus Capsid Protein: Role in Early Replication Events

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The Rubella virus capsid protein is phosphorylated prior to virus assembly. Our previous data are consistent with a model in which dynamic phosphorylation of the capsid regulates its RNA binding activity and, in turn, nucleocapsid assembly. In the present study, the process of capsid phosphorylation was examined in further detail. We show that phosphorylation of serine 46 in the RNA binding region of the capsid is required to trigger phosphorylation of additional amino acid residues that include threonine 47. This residue likely plays a direct role in regulating the binding of genomic RNA to the capsid. We also provide evidence which suggests that the capsid is dephosphorylated prior to or during virus budding. Finally, whereas the phosphorylation state of the capsid does not directly influence the rate of synthesis of viral RNA and proteins or the assembly and secretion of virions, the presence of phosphate on the capsid is critical for early events in virus replication, most likely the uncoating of virions and/or disassembly of nucleocapsids.

Rubella virus (RV) is the sole member of the genus *Rubivirus* within the family *Togaviridae*. In most cases, RV infection causes a mild self-limiting disease in humans known as rubella or German measles. However, in contrast to postnatal infection, which is relatively benign in nature, in utero infection can have serious consequences for the developing fetus (6). When contracted during the first trimester of pregnancy, RV infection results in a characteristic series of severe defects in the neonate known as congenital rubella syndrome. Despite the availability of an effective vaccine, RV remains a threat to public health in both developed and developing countries as a result of unvaccinated populations. The mechanisms by which RV causes developmental abnormalities are largely unknown.

As with all togaviruses, the RV genome contains two open reading frames that encode the replicase proteins and the structural proteins (reviewed in reference 11). The structural proteins are translated as a polyprotein precursor from a subgenomic RNA. The precursor protein is then processed into three structural proteins: a capsid protein and two envelope glycoproteins, E2 and E1. Recently, our laboratory has focused largely on the processing and functional analyses of the capsid protein.

During virus assembly, the capsid is thought to interact with viral genomic RNA and glycoproteins E2 and E1 to drive formation of the nucleocapsid and virus budding, respectively. Nucleocapsid formation is highly regulated in RV-infected cells and is coincident with virus budding (29, 32). The packaging signal within the RV genomic RNA and the capsid region that binds this sequence have been identified (24). Moreover, data from our lab suggest that phosphorylation of serine 46 within the RNA binding site of the capsid regulates interactions between the capsid and genomic RNA and, by extrapolation, nucleocapsid formation (22). Mutation of serine 46 to alanine results in capsids that are poorly phosphorylated and exhibit relatively high RNA binding activity. Furthermore, virus strains that express hypophosphorylated capsids replicate at lower titers and are less cytopathic than wild-type virus, suggesting that phosphorylation of the capsid is an important event in the virus life cycle. We proposed that capsid phosphorylation is a dynamic process whose initial function is to prevent premature binding of genomic RNA. Conversely, phosphorylation of the capsid may be involved in virion disassembly and release of genomic RNA from nucleocapsids. In this study, we further examined the regulation of capsid phosphorylation and its role in the virus life cycle.

MATERIALS AND METHODS

Reagents, antibodies, and cDNA clones. Reagents and supplies were from the following sources. Protein A- and G-Sepharose were purchased from Pharmacia (Alameda, CA). Phenylmethylsulfonyl fluoride, fibronectin, sodium dodecyl sulfate (SDS), bovine serum albumin, and general lab chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). ^{14}C-labeled protein standards were purchased from Amersham Corp. (Arlington Heights, IL). Radiolabeled inorganic phosphates $H_3{}^{33}PO_4$ (10 mCi/ml) and $H_3{}^{32}PO_4$ (5 mCi/ml) were purchased from ICN (Costa Mesa, CA). Media and sera for cell culture were purchased from Life Technologies-Invitrogen, Inc. (Carlsbad, CA). PerFectin transfection reagent was purchased from Gene Therapy Systems, Inc. (San Diego, CA). Vero, COS, and BHK-21 cells were obtained from the American Type Culture Collection (Manassas, VA). The C1 anticapsid and the B2 anti-E1 monoclonal antibodies were kind gifts from Jerry Wolinsky (University of Texas, Houston) and Barbara Pustowoit (University of Leipzig, Germany), respectively. Rabbit polyclonal antibodies to the nonstructural protein p150 were kindly provided by Tero Ahola (University of Helsinki). Rabbit anticalnexin was purchased from StressGen Biotechnology Corporation (Victoria, British Columbia, Canada). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G and goat anti-rabbit immunoglobulin G were purchased from Bio-Rad (Hercules,

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TABLE 1. Oligonucleotide primers

Primer name	Sequence (5′ to 3′)[a]	Underlined sequence	Mutation(s)
CapSal(R)	GTAAAATGCAGGTCGACG	SalI	None
P5D(F)	CCGCGGCCGCCGCGACAGCGCGAC**GACGACGACGAC**GGAGATGAC**GAC**GGCCGTGACTCC	NotI	S45>D, S46>D, T47>D, S48>D, S52>D
P3D(F)	CCGCGGCCGCCGCGACAGCGCGAC**GACGACGAC**TCCGGAGATGACTCCGGCCGTGACTCC	NotI	S45>D, S46>D, T47>D
P3E(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**GAAGAAGAA**GGAGATGACTCCGGCCGTGACTCC	NotI	S46>E, T47>E, S48>E
P3A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGCAGCC**TCCGGAGATGACTCCGGCCGTGACTCC	NotI	S46>A, S46>A, T47>A
S46N(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**AAC**ACCTCCGGAGATGACTCCGGCCGTGACTCC	NotI	S46>N
S46D(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**GAC**ACCTCCGGAGATGACTCCGGCCGTGACTCC	NotI	S46>D
S46E(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**GAA**ACCTCCGGAGATGACTCCGGCCGTGACTCC	NotI	S46>E
T47A(F)	CCGCGGCCGCCGCGACAGCGCGACTCCAGC**GCC**TCCGGAGATGACTCCGGCCGTGACTCC	NotI	T47>A
T47E(F)	CCGCGGCCGCCGCGACAGCGCGACTCCAGC**GAA**TCCGGAGATGACTCCGGCCGTGACTCC	NotI	T47>E

[a] Mutagenic residues are in bold.

CA). The M33 strain of RV and pBRM33, the infectious RV cDNA clone (39), were kindly provided by Shirley Gillam (University of British Columbia).

Mammalian cell culture. COS, BHK-21, and Vero cells were cultured in Dulbecco's minimal essential medium (DMEM) (high glucose) containing 10% fetal bovine serum, 2 mM glutamine, 1 mM HEPES, and antibiotics. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Plasmid construction. Capsid phosphorylation mutants (pCMV5-CapP5D, -CapP3D, -CapP3A, -CapS46N, -CapS46D, -CapS46E, and -CapT47A) were constructed as described previously for pCMV5-CapS46A (22). Briefly, capsid mutants were generated by PCR using *Taq* polymerase and pCMV5-E2SP (21) as the template. Forward primers encoded site-specific mutations, and CapSal(R) was used as the reverse primer (primer sequences are listed in Table 1). Resulting PCR products were digested with NotI and SalI, purified, and then used to replace the 401-base-pair fragment of pCMV5-CapE2SP. All new cDNA constructs were sequenced to verify their authenticity and ensure the absence of second-site mutations.

Transfection of mammalian cells. COS cells (1.5×10^5) in 35-mm-diameter culture dishes were transfected with 2 μg of each plasmid combined with 7 μl of PerFectin transfection reagent as described by the manufacturer. Cells were incubated in culture medium for 24 h prior to biosynthetic labeling experiments or for 48 h prior to use in immunoprecipitation experiments.

Synthesis of infectious viral RNA and production of recombinant viruses. The RV infectious clone pBRM33 (39) and the S46A mutant (22) were described previously. Infectious viral RNAs were used for electroporation of BHK-21 cells, and the resulting secreted virions were harvested and propagated as described previously (22). Where indicated, Vero cells were transfected with approximately 3 μg of infectious viral RNA complexed with Lipofectamine-2000 transfection reagent (Gibco/BRL) as described previously (35). The levels of secreted infectious virus and total secreted virus antigen were determined by plaque assay and immunoblot analysis, respectively. For immunoblot analysis, crude virions were isolated by using a two-step centrifugation protocol. Briefly, conditioned medium from infected cells was precleared of cellular material by centrifugation at $10{,}000 \times g$ for 10 min. Virions were recovered from the resulting supernatants by centrifugation at $100{,}000 \times g$ for 60 min.

Infection of Vero cells with RV. Virus stocks were diluted with cell culture medium and overlaid onto phosphate-buffered saline (PBS)-washed cell monolayers (approximately 1 ml/35-mm dish) for a minimum of 4 h at 35°C. The virus inoculum was replaced with normal growth medium, and the cells were cultured at 35°C until processing for experimental analyses.

Radiolabeling of capsid and immunoprecipitation. Where indicated, transfected COS or RV-infected Vero cells were radiolabeled with phosphorus-32 ($H_3{}^{32}PO_4$) or phosphorus-33 ($H_3{}^{33}PO_4$). Prior to labeling, cells in 35-mm dishes were washed once in PBS, incubated in phosphate-free DMEM for at least 30 min, and then labeled in phosphate-free DMEM containing 100 μCi radioactive phosphate for 4 to 16 h.

Cell-associated capsid proteins were purified from radiolabeled transfected COS cells by immunoprecipitation as described previously (22). To purify capsid protein from virions, radiolabeled virions were immunoprecipitated from medium as follows. Media from infected Vero cells were precleared by centrifugation at $14{,}000 \times g$ for 10 min at 4°C prior to immunoprecipitation with 5 to 10 μg of monoclonal mouse anti-E1 antibody (B2) and protein G-Sepharose (25 μl) at 4°C. The immunocomplexes were washed with PBS to preserve the integrity of the virions. During immunoprecipitation, all solutions contained phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM tetrasodium pyrophosphate).

Phosphopeptide mapping. ^{32}P-labeled wild-type and P3D mutant capsid proteins were isolated from transfected COS cells (100-mm dish) by immunoprecipitation with rabbit anticapsid antibodies. Typically, cells were labeled overnight in phosphate-free medium containing 2 mCi of $H_3{}^{32}PO_4$. Samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and after autoradiography, regions of the gels containing capsid were excised. Radiolabeled capsid proteins were eluted from the gel slices by addition of 500 μl of freshly prepared 50 mM ammonium bicarbonate (pH 7.3) to which 50 μl of β-mercaptoethanol and 10 μl of 10% SDS were added. Samples were incubated overnight at 37°C, after which the supernatants were precipitated with trichloroacetic acid in the presence of bovine serum albumin as a carrier protein (20 μg). Next, proteins were oxidized with a 9:1 mixture of 98% formic acid and 33% hydrogen peroxide at 0°C for 60 min. The performic acid was evaporated by lyophilization, and the proteins were resuspended in 50 μl of ammonium bicarbonate (pH 8.0) containing 10 μg of TPCK (tosylamido-2-phenylethyl chloromethyl ketone)-trypsin. Samples were incubated overnight at 37°C.

After digestion, the samples were repeatedly washed with deionized water and lyophilized to remove any ammonium bicarbonate and insoluble proteins. Finally, the tryptic peptide mixture was resuspended in a buffer (10 μl) containing 2.2% formic acid and 7.8% glacial acetic acid (pH 1.9). Samples were spotted onto 20- by 20-cm cellulose thin-layer chromatography plates. The first dimension, the thin-layer electrophoresis, was performed at 1.0 kV for 25 min in buffer containing 2.2% formic acid and 7.8% glacial acetic acid (pH 1.9). The plates were then air dried overnight, after which ascending chromatography was performed in *n*-butyl alcohol–pyridine–glacial acetic acid–water (15:10:3:12, vol/vol/vol/vol). The plates were air dried, and phosphopeptides were detected by using a phosphorimager.

In vitro RNA binding assay and Northern blotting. RNA binding assays were performed as described previously (22), except that where indicated, the probe was labeled with cytidine [α-^{32}P]triphosphate (3,000 Ci/mmol; ICN) instead of cytidine [α-^{35}S]triphosphate. RNA extraction and Northern blotting, using NR-6622-3′ as a probe, was conducted as previously described (1).

RESULTS

Capsid is phosphorylated at multiple sites. We previously demonstrated that phosphorylation of the RV capsid protein regulates its binding to genomic RNA (22). The serine residue at position 46 was shown to be important for regulating the overall phosphorylation state of the capsid. The introduction of point mutations in the capsid gene that result in changing serine 46 to alanine (S46A) effectively block capsid phosphorylation and negatively impact virus replication. While these studies clearly established the importance of serine 46 in capsid phosphorylation, it was not determined whether additional amino acid residues were subject to phosphorylation. Therefore, we elected to examine the characteristics of mutants that mimic constitutive phosphorylation at serine 46 of the capsid. Initially, serine 46 was replaced with the acidic amino acid residues aspartate or glutamate to create CapS46D or

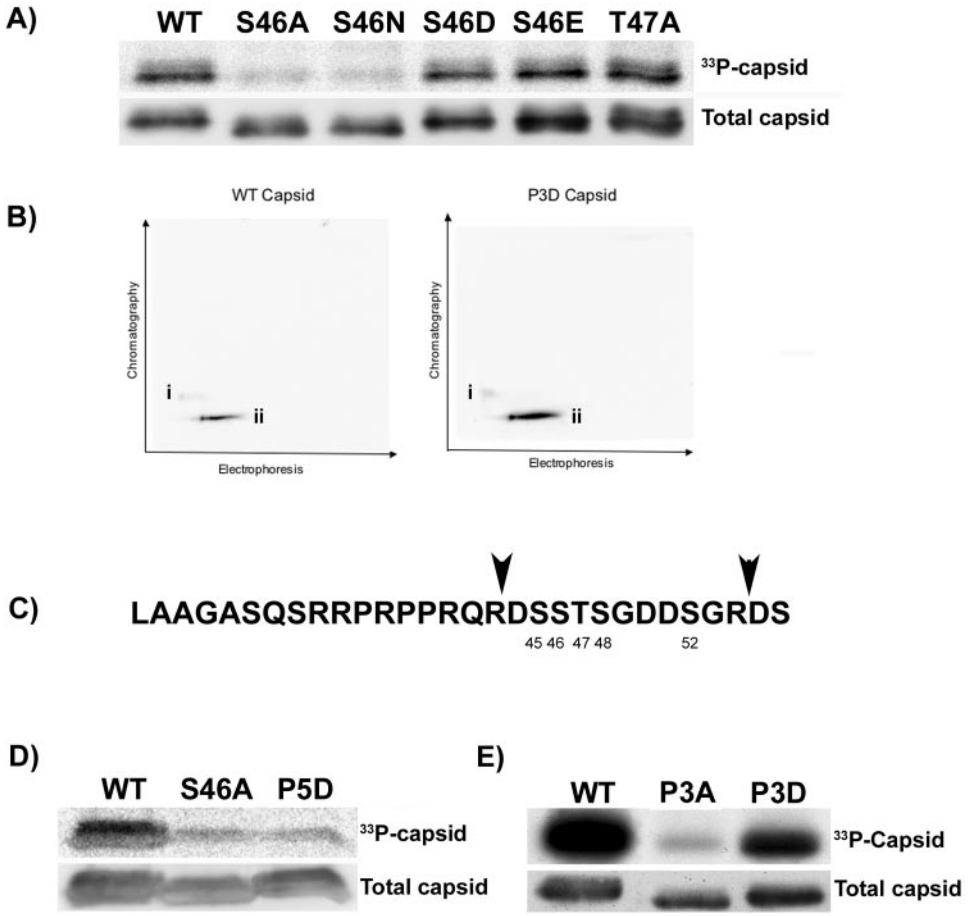

FIG. 1. Substitution of acidic amino acid residues at position 46 allows progressive phosphorylation of the capsid. (A, D, and E) COS cells were transiently transfected with expression vectors encoding wild-type (WT) or mutant capsid constructs. Twenty-four hours posttransfection, cells were incubated with medium containing [^{33}P]orthophosphate for 12 h prior to lysis. Samples were subjected to radioimmunoprecipitation with rabbit anticapsid serum, SDS-PAGE, and fluorography (upper panels). Relative expression levels of capsid proteins were determined by probing the same membranes with a monoclonal antibody to capsid followed by detection using enhanced chemiluminescence (lower panels). Experimental conditions were identical for panels A, D, and E. (B) ^{32}P-labeled wild-type and P3D capsid proteins were subjected to tryptic peptide mapping. The directions of separation by electrophoresis and chromatography are indicated. Minor (i) and major (ii) phosphopeptides were resolved in both cases. The sequence of the capsid RNA binding region is shown using the one-letter amino acid code in panel C. Serine 46 and other potentially phosphorylated amino acid residues are indicated by numbers under the sequence of the RNA binding region. Trypsin cleavage sites bounding the predicted phosphoamino acid cluster are indicated by arrowheads. In the P3A and P3D mutants, serines 45 and 46 and threonine 47 were changed to alanines and aspartic acid residues, respectively. For P5D, serines 45, 46, 48, and 52 and threonine 47 were changed to aspartate.

CapS46E, respectively. The size and charge of the carboxyl side chains on aspartate and glutamate are such that they have been used by other laboratories to successfully mimic phosphoserine and phosphothreonine (19, 38, 41). The mutant capsid proteins CapS46D and CapS46E were both heavily phosphorylated, although not to the same extent as wild-type capsid (Fig. 1A). Quantitation of the data revealed that CapS46D and CapS46E incorporated approximately 20% less radioactive phosphate than wild-type capsid. In contrast, substitution of an asparagine at position 46 (CapS46N) effectively blocked phosphorylation of the capsid. Similar to the CapS46A mutant, CapS46N incorporated approximately seven times less phosphate than wild-type capsid. Together, these results indicate that in addition to serine 46, the capsid is phosphorylated at other sites. However, phosphorylation of serine 46 appears to be required for triggering subsequent phosphorylation events. In this respect, the negative charge on aspartate and glutamate residues is sufficient to mimic the phosphate group that is normally added to serine 46.

To determine which amino acid residues are involved in serine 46-dependent phosphorylation events, other capsid gene codons that encode potentially phosphorylated serine/threonine residues were mutated. Fortunately, we were able to narrow down the number of potentially phosphorylated amino acid residues in the capsid and consequently the number of codons that were to be altered by site-directed mutagenesis. First, our previous studies indicated that the majority of capsid phosphorylation occurs in the amino-terminal portion of the protein, a region that includes the RNA binding site (22). Second, data from tryptic peptide mapping suggest that the major phosphoamino acid residues are clustered within a single peptide (Fig. 1B). This major phosphopeptide contains over 99% of the phosphate associated with the capsid, and therefore, it is likely that serine 46 is located within this pep-

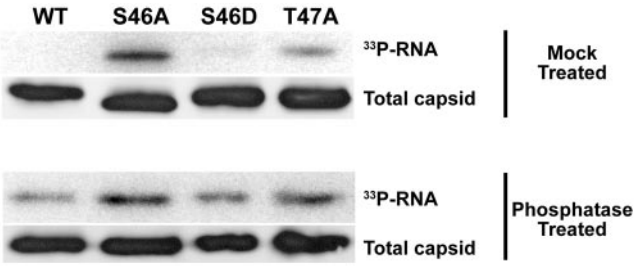

FIG. 2. Mutations at position 47 affect the RNA binding activity of the capsid. Capsid proteins were isolated from transfected COS cells by immunoprecipitation, treated with phosphatase or mock treated, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated with ^{33}P-labeled RV genomic RNA and washed, and RNA binding to the capsid was detected by using a phosphorimager (upper panels). Relative capsid expression levels were determined by stripping the membranes and immunoblotting with a monoclonal antibody to capsid (lower panels).

tide. For these reasons, we limited our mutational analysis to the RNA binding region of the capsid (Fig. 1C). Initially, we examined the phosphorylation state of CapP5D, a capsid mutant in which serine residues 45, 46, 48, and 52 and threonine 47, all within the RNA binding site, were replaced by aspartate residues. This construct exhibited a low level of phosphorylation similar to hypophosphorylated capsids such as CapS46A (Fig. 1D). Since the substitution of aspartate at position 46 effectively induces phosphorylation of other amino acid residues in the capsid, the low level of phosphorylation exhibited by CapP5D indicates that most if not all of the major phosphorylated amino acid residues of the capsid reside within the RNA binding site. Together, these results suggest that the phosphorylation of serine 46 allows one or more of the following residues to be phosphorylated: serine 45, 48, or 52 or threonine 47. However, because we previously demonstrated that mutation of serine 45 does not affect phosphorylation of the capsid (22), it is likely that one or more of the following three residues are phosphorylated: serine 48 or 52 or threonine 47. Furthermore, since CapP3D is heavily phosphorylated (Fig. 1E) despite the fact that the potential phosphorylation sites at positions 45, 46, and 47 have been eliminated in this construct, it is likely that serine residues 48 and 52 are major phosphorylation sites. Finally, because the phosphopeptide profile of P3D is identical to that of wild-type capsid (Fig. 1B), we do not think that this mutant is abnormally phosphorylated at amino acid residues that lie outside the RNA binding site. Together, these results suggest that the major phosphoamino acid residues within the capsid are confined to a single tryptic peptide (DSSTSGDDSGR).

RNA binding activity of the capsid is not directly regulated by phosphorylation of serine 46. As mentioned above, recent experiments from our laboratory revealed that phosphorylation of the capsid negatively regulates its binding to genomic RNA (22). We next investigated whether mutations that mimic constitutive phosphorylation within the RNA binding site of the capsid would be sufficient to inhibit this interaction. The CapS46D mutant exhibited relatively low RNA binding activity in vitro (Fig. 2, upper panels). This result was not unexpected, because this protein is heavily phosphorylated at sites other than serine 46 (Fig. 1A). To directly assess how phosphorylation at these other sites affects RNA binding, CapS46D was treated with phosphatase prior to the in vitro RNA binding assay. After phosphatase treatment, CapS46D bound genomic RNA as well as dephosphorylated wild-type capsid (Fig. 2, lower panels). These results indicate that phosphorylation of amino acid residues other than serine 46 is important for regulating interaction between capsid and genomic RNA.

Although the replacement of serine with acid amino acid residues at position 46 was able to substitute for phosphoserine in triggering the phosphorylation of other amino acid residues in the capsid, the substituted amino acid residues were not able to fully mimic the function of phosphate in regulating RNA binding. This phenomenon is illustrated by the properties of the CapP5D mutant, in which serine and threonine residues at positions 45, 46, 47, 48, and 52 were replaced with aspartate (Table 2). The CapP5D mutant was designed to mimic fully phosphorylated capsid protein but is in fact minimally phosphorylated (Fig. 1D). The observation that it still binds genomic RNA (Table 2) indicates that aspartate residues do not mimic the effect of phosphorylation in blocking the capsid-RNA interaction.

To further understand the role of individual phosphoamino acid residues in regulating RNA binding, additional capsid constructs with point mutations in the RNA binding region were analyzed. Most of the capsid mutants exhibited a common trend with respect to phosphorylation status and RNA binding: if capsid constructs were poorly phosphorylated, they bound RNA, and if they were heavily phosphorylated, they did not bind RNA well (Table 2). However, CapP3D is an exception to this trend. This protein incorporated more phosphate than other hypophosphorylated mutants, such as CapS46A, CapS46N, CapP3A, and CapP5D (Fig. 1D and E), but it was still able to efficiently bind genomic RNA (Table 2). Based on these results, we conclude that serine 48, serine 52, or both are phosphorylated and that phosphorylation of these amino acid residues does not inhibit the RNA binding activity of the capsid.

The relatively high RNA binding activity of CapP3D indicates that blocking phosphorylation at serines 45 and 46 and/or threonine 47 allows RNA binding. Based on the evidence pre-

TABLE 2. Characteristics of intracellular capsid proteins

Construct	Mutation(s)	Relative phosphorylation[a]	RNA binding[b]
Wild type	None	+++++	−
S46A	S46>A	+	++
S46N	S46>N	+	++
S46D	S46>D	++++	−
S46E	S46>E	++++	−
T47A	T47>A	++++	+
P3A	S45>A, S46>A, T47>A	+	++
P3D	S45>D, S46>D, T47>D	+++	++
P5D	S45>D, S46>D, T47>D, S48>D, S52>D	+	+

[a] +++++, phosphorylation level observed in the wild-type intracellular capsid; +++ or ++++, phosphorylation level is 50 to 80% of that observed in the wild-type capsid; +, minimal phosphorylation (<20% of that seen in the wild-type intracellular capsid).

[b] ++, high RNA binding capacity; +, low RNA binding capacity; −, no detectable RNA binding. RNA binding activities are those reported for mock-treated intracellular capsids.

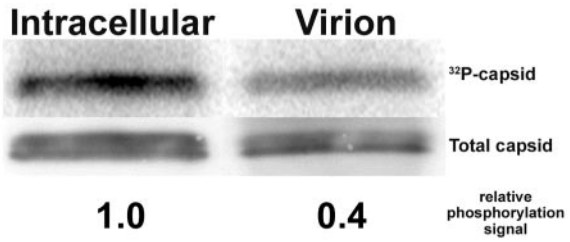

FIG. 3. Virion-associated capsids contain less phosphate than cell-associated capsids. Vero cells cultured in the presence of [^{32}P]orthophosphate were infected with M33 RV (MOI = 10). Three days postinfection, intracellular and virion-associated capsids were isolated by immunoprecipitation and subjected to SDS-PAGE. Phosphorylated capsids were detected and quantitated using a phosphorimager (upper panel), and the values were normalized to the total capsid levels determined by immunoblotting (lower panel). The normalized phosphorylation level of intracellular capsid was set to 1.0. Values are the average of results from two independent experiments.

sented in Fig. 2 and Table 2, we do not expect serines 45 or 46 to be directly involved in RNA binding. Moreover, our previous studies suggest that serine 45 is not phosphorylated (22). Therefore, the most logical conclusion from these data is that phosphorylation of threonine 47 directly inhibits RNA binding. To address this possibility, threonine 47 was replaced with alanine to create the mutant CapT47A. This construct exhibited higher RNA binding activity than the wild-type capsid or CapS46D (Fig. 2, upper panels), despite being heavily phosphorylated (Fig. 1A). Moreover, phosphatase treatment did not dramatically increase the RNA binding activity of this protein (Fig. 2). These results indicate that blocking phosphorylation at threonine 47 is sufficient to allow RNA binding to the capsid irrespective of the overall phosphorylation state of the protein.

Virion-associated capsid contains less phosphate than cell-associated capsid. Based on the data described above and previously (22), we propose that phosphorylation of capsid protein early in the assembly pathway is required to prevent premature assembly of the nucleocapsid and/or to minimize the interaction of capsid with cellular RNAs. Conversely, nucleocapsid assembly would require timely dephosphorylation of the capsid. To determine whether the capsid is in fact dephosphorylated before or during packaging into virions, the phosphorylation levels of intracellular and virion-associated capsids were compared. Cells infected with the wild-type M33 strain of RV were grown in the presence of [^{32}P]orthophosphate. Three days postinfection, intracellular and virion-associated capsids were immunoaffinity purified and then subjected to SDS-PAGE and fluorography. The phosphorylation levels of the two capsid pools were normalized to the amounts of total capsid determined by immunoblotting. The virion-associated capsids were found to contain less than half the level of phosphate associated with intracellular capsids (Fig. 3).

Capsid phosphorylation is important for early steps in virus replication. Results from both in vitro and in vivo experiments indicate that phosphorylation of the capsid negatively regulates the capsid-RNA interaction. Dephosphorylation may be a molecular switch that allows the capsid to interact with the genomic RNA, a process that is required for the formation of the nucleocapsid and subsequent virus budding. Conversely, timely rephosphorylation of the capsid may also be expected to decrease its RNA binding activity in order to facilitate nucleocapsid disassembly following endocytosis. If this hypothesis has merit, nucleocapsid disassembly is expected to occur less efficiently in cells infected with the S46A strain. In turn, this would be expected to delay and/or decrease the expression of viral proteins and RNA because the genomic RNA would not be translated and/or transcribed as quickly or as efficiently. To address this scenario, we compared the expression profiles of nonstructural proteins (p150), structural proteins (capsid), and virus-specific RNAs in cells infected with wild-type (M33) and phosphorylation mutant (S46A) viruses. Both p150 and capsid were seen to accumulate at earlier time points in lysates of cells infected with M33 virus (Fig. 4A and B). At a high multiplicity of infection (MOI = 5), p150 was detectable by 16 h postinfection in M33-infected cells and steadily accumulated until 36 h postinfection. In contrast, the expression of p150 was not detected until 36 h postinfection in cells infected with the S46A mutant virus (Fig. 4A). Although capsid was detected at earlier time points, the buildup of this antigen from de novo synthesis followed a trend similar to that of p150 (Fig. 4A). Specifically, capsid accumulated more rapidly in cells infected with M33 than with S46A mutant virus. The appearance of capsid at early time points, even prior to accumulation of p150, may be explained by two not mutually exclusive reasons: (i) immunoblotting with the anticapsid antibodies is more sensitive than that with the anti-p150 antibodies and/or (ii) this pool of capsid originates from nucleocapsids of incoming virions. The latter possibility is further supported by the fact that capsid was not detected at early time points when a lower MOI was used (Fig. 4B) or when the viral genome was delivered to cells by transfection (Fig. 5A).

The kinetics of genomic and subgenomic RNA accumulation were also monitored by Northern blot analysis. Similar to the Western blot results, a more rapid buildup of the viral RNAs was observed in cells infected with the wild-type virus than in cells infected with the S46A mutant virus (Fig. 4C). These differences were independent of the amount of virus present during infection, as evidenced by the fact that similar results were obtained when the experiments were performed at both high and low MOIs (Fig. 4C, compare upper and lower panels). These results indicate that both the transcription of the viral RNAs and the synthesis of viral proteins are negatively affected in cells infected with the S46A mutant virus and together are consistent with a defect in nucleocapsid disassembly. In addition to delaying the exposure of viral genomes to the host translation apparatus, the inability to efficiently disassemble nucleocapsids may ultimately result in fewer genomes being available for translation.

Based on recent reports which pointed to a role for the capsid in regulating viral transcription, it has been suggested that proper phosphorylation of the capsid is required for enhancing virus replication (8, 34). Therefore, it was essential to determine whether the delay in accumulation of viral proteins and RNAs was a direct result of the inability of the capsid to be phosphorylated. In order to test this possibility, an experiment similar to that described above was performed, except that transfection was used to directly deliver the viral genomes to Vero cells. This method bypassed the step of virus uncoating and therefore removed nucleocapsid disassembly as a factor.

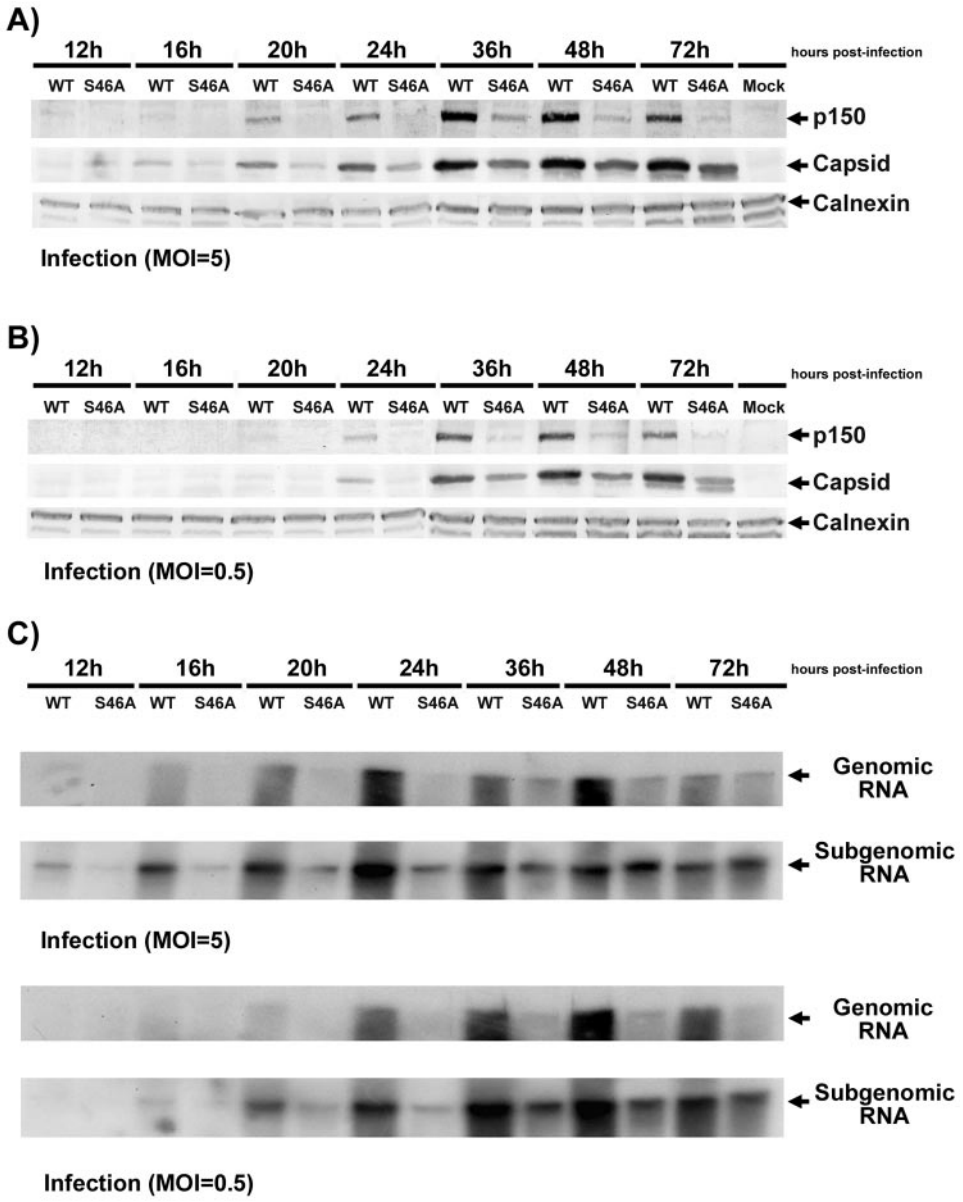

FIG. 4. Synthesis of virus-encoded proteins and RNA is reduced in S46A mutant-infected cells. Vero cells were infected with either wild-type M33 or the S46A strain virus at an MOI of 5 (A and C, upper panels) or an MOI of 0.5 (B and C, lower panels). At regular intervals after infection, cell lysates were prepared and subjected to SDS-PAGE and immunoblotting with antibodies to p150 and capsid (A and B). The membranes were also probed with anticalnexin antibodies as a control for protein loading (A and B). (C) Total RNA was isolated from cells infected with the M33 or S46A strain, and the levels of RV genomic and subgenomic RNAs were determined by Northern blotting.

Equal amounts of viral transcripts encoding either M33 or the S46A mutant virus were transfected into Vero cells, and the expression of virus proteins and subgenomic RNA in transfected cells was monitored as described above. Regardless of whether cells were transfected with RNAs derived from M33 or the S46A mutant virus, both capsid and p150 accumulated with similar kinetics (Fig. 5A). In addition, the levels of subgenomic RNA in the transfected cells were similar at early and middle time points (12 to 24 h) (Fig. 5B). Together, these data indicate that mutation of the capsid gene does not directly affect translation or transcription of the virus genome. These results are in agreement with a recent study by members of this group which showed that phosphorylation of the capsid is not important for its ability to modulate the ratio of genomic and subgenomic RNAs (36).

Phosphorylation of capsid protein is not important for virion secretion. Previously, we determined that cells infected with strains of RV with mutations at codon 46 of the capsid gene secrete 10- to 60-fold less infectious virus than cells infected with the wild-type M33 strain of RV (22). Although viral RNA and protein synthesis are not directly affected by S46 mutations, it is still possible that virion assembly and/or secretion are impaired in S46A mutant-infected cells. To examine this potential scenario, cells that had been electroporated with

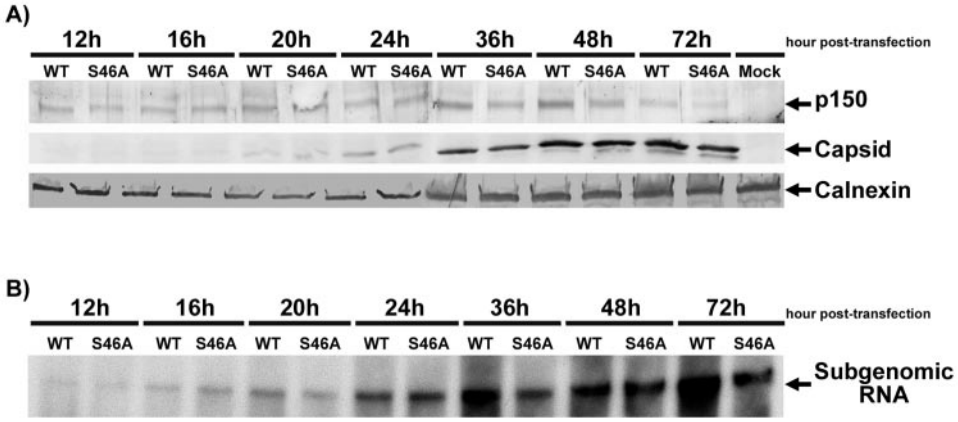

FIG. 5. Direct delivery of the S46A mutant genome to Vero cells by electroporation abrogates the early replication defects. Equal amounts of genomic RNA were delivered into Vero cells by transfection. (A) At various time points posttransfection, the levels of virus proteins p150 (upper panel) and capsid (middle panel) were determined. The membranes were also probed with anticalnexin antibodies as a control for protein loading. (B) The levels of subgenomic RNA were also determined as described in the legend to Fig. 4.

M33- and S46A mutant-specific genomic RNAs were assayed for virion secretion. Medium samples were removed at regular times postelectroporation, and crude virion preparations were isolated by a two-step centrifugation method (12). The presence of capsid protein in the medium pellet fractions is indicative of secretion of virions. The results shown in Fig. 6 show that the levels of virus particle secretion were similar (±20%) at all times for the M33 and S46A mutant samples. Immunoblot analysis of the cell lysates confirmed that similar levels of capsid protein were produced in each case (Fig. 6). These results indicate that preventing capsid phosphorylation does not significantly affect virion assembly and secretion. Collectively, our data suggest that the replication defects associated with the S46A mutant virus result from inefficient nucleocapsid disassembly, which in turn causes a delay and/or inhibition of viral RNA and protein expression.

Virion-associated capsid does not change its phosphorylation state after entering the host cell. The fact that early replication defects are observed with the S46A mutant virus is consistent with the notion that capsid phosphorylation is important for disassembly of the nucleocapsid. One possible mechanistic explanation is that the incoming virion-associated capsids are phosphorylated in order to lower their RNA binding affinity, thereby facilitating the release of viral genomes. As the S46A mutant capsid cannot be properly phosphorylated, this may account for the delay in the expression of viral proteins and subgenomic RNA in infected cells. To further investigate the role of capsid rephosphorylation in virion disassembly, we examined the phosphorylation state of the capsid during the initial stages of infection. Vero cells infected with M33 RV (MOI = 10) were cultured in the presence of [^{32}P]orthophosphate, and capsids were immunopurified from cell lysates at regular intervals. The immunopurified capsids were then separated by SDS-PAGE and processed for fluorography.

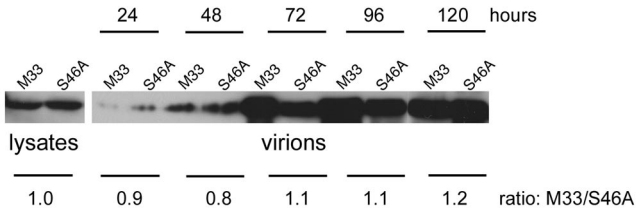

FIG. 6. The S46A mutation in the capsid gene does not affect virion assembly. BHK cells were electroporated with 5 μg of in vitro-synthesized capped RNAs specific for the M33 and S46A RV strains. Medium samples were collected at the indicated times (in hours) postinfection, and virions were isolated by two-step centrifugation. Samples were analyzed for the presence of capsid protein by immunoblotting. Equivalent aliquots of cell lysates (120 h postinfection) were also subjected to immunoblotting analysis with antibodies to capsid. The ratio of M33/S46A capsid is shown at each time point.

In agreement with a previous study (15), de novo synthesis of capsid protein was observed between 12 and 20 h postinfection. This process was evidenced by the sharp increase in capsid levels at 20 h postinfection (Fig. 7A, lanes 6 and 7, lower panel). Phosphorylation of the nascent capsid was readily detected at the same time points (Fig. 7A, lanes 6 and 7, upper panel). In contrast, capsid derived from incoming viruses did not incorporate detectable amounts of ^{32}P (Fig. 7A, lanes 1 to 5). This observation suggests that incoming capsid proteins do not undergo rephosphorylation during the uncoating of virions. In order to eliminate doubt that the lack of detectable phosphorylation of incoming capsids is due to the relatively low levels of this protein during early infection, the experiment was repeated by scaling up the amount of infected cells in an effort to collect enough capsid protein from the incoming virions (4 h postinfection). In addition, samples were diluted from the pool of phosphorylated capsid during the later stages of infection (30 h postinfection) to show that phosphorylated capsid can be detected among relatively small quantities of capsid. In Fig. 7B (lanes 3 and 4), it can be seen that whereas the levels of total capsid were at the limits of detection by immunoblotting, ^{32}P-labeled capsid was readily detected at these time points. In contrast, despite the relatively large amounts of capsid protein collected at 4 h postinfection, phosphorylated capsids were not detected in this pool (Fig. 7B, lane 2). Together, these results indicate that the capsid protein from

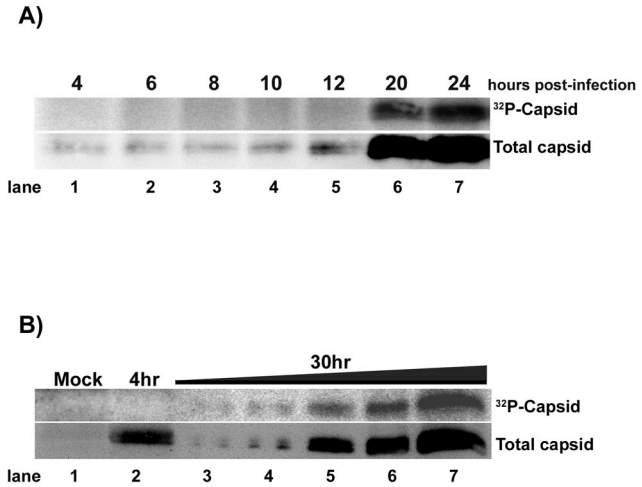

FIG. 7. Incoming capsids are not rephosphorylated. (A) Vero cells were infected with the M33 strain of RV (MOI = 10) in the presence of [^{32}P]orthophosphate. At regular intervals postinfection, intracellular capsids were isolated by immunoprecipitation. Samples were then subjected to SDS-PAGE and fluorography (upper panel). Relative capsid levels were determined by probing membranes with a mouse monoclonal antibody to capsid followed by enhanced chemiluminescence detection (lower panel). (B) Vero cells were infected with the M33 strain of RV (MOI = 10). RV-infected cells were labeled with [^{32}P]orthophosphate for 3 h at either 4 h postinfection (lane 2) or 30 h postinfection (lanes 3 to 7). Intracellular capsids were then isolated by immunoprecipitation. In order to collect relatively large amounts of capsid protein from incoming virions, twice the amount of cell lysate used in panel A was used for immunoprecipitation. Increasing amounts of the sample collected at 30 h postinfection were loaded to illustrate that the phosphate signal can be detected among relatively small amounts of phosphorylated capsid (lanes 3 to 7).

incoming virions is not rephosphorylated during nucleocapsid disassembly.

DISCUSSION

Phosphorylation is one of many ways to modulate the functions of virus proteins during replication and assembly. Indeed, numerous studies indicate that phosphorylation of capsid proteins is essential for successful virus infection (5, 7, 16, 19, 20, 23, 26, 28, 31, 40). While in many cases the specific roles of capsid phosphorylation have yet to be established, this posttranslational modification is known to affect genome packaging (9, 13, 16, 17, 20) and subcellular localization of capsid proteins (18, 19, 25, 30). Interestingly, it was first reported more than 30 years ago that togavirus capsid proteins are phosphorylated (27, 37). However, surprisingly little is known about which amino acid residues in the capsids are modified and how phosphorylation regulates the functions of these proteins. In this study, we identified several phosphorylated amino acid residues that are critical for regulating RV capsid phosphorylation and virus replication.

Identification of RV capsid phosphorylation sites. The RV capsid is phosphorylated on multiple sites, with the majority of or all phosphorylated amino residues concentrated within the RNA binding site. Of these residues, serines 46, 48, and 52 and threonine 47 are likely to be the major phosphorylated amino acid residues. Serine 46 is the most important amino acid residue with respect to regulating phosphorylation of the capsid (22), as phosphorylation of this residue is necessary for subsequent phosphorylation of other amino acid residues within the RNA binding site. Since acidic residues (aspartate or glutamate) at position 46 can mimic the effect of phosphorylated serine in terms of allowing downstream phosphorylation, it is likely that the negative charge at this location triggers an as yet unidentified kinase to phosphorylate other amino acid residues.

The function of capsid phosphorylation in virus replication. Although phosphorylation of the capsid is important for virus replication, assembly of virions is not dependent upon this process. Rather, viruses that encode hypophosphorylated capsid proteins have early replication defects, possibly due to problems with disassembly of nucleocapsids. This observation is in agreement with our proposed model that capsid phosphorylation is dynamic, a situation that would facilitate both the efficient binding and the release of viral RNA at the appropriate points in the replication cycle. Indeed, data presented here are consistent with a scenario in which capsid proteins undergo a dephosphorylation step before they are packaged into virions. However, it cannot be distinguished at this point whether all capsid proteins undergo dephosphorylation to the same degree or whether only a fraction of the capsid pool becomes completely dephosphorylated. Conversely, we predicted that phosphorylation of the capsid by a cellular kinase destabilizes the nucleocapsid to release the genomic RNA during virus entry. This process would presumably afford easier access of ribosomes to the genomic RNA, allowing translation of the nonstructural proteins to occur with greater efficiency. For example, encapsidated RNA of potato virus X is nontranslatable in vitro but can be rendered translatable after phosphorylation of the coat protein (2). In the case of RV, however, we were unable to detect a change in the level of capsid phosphorylation during the early stages of infection.

Why are expression and/or accumulation of RV proteins and RNA inhibited in cells infected with viruses that encode hypophosphorylated capsid proteins? The most logical and simplest explanation is that the intrinsic high affinity of hypophosphorylated capsid (S46A) for RNA delays nucleocapsid disassembly. In wild-type virions, the level of capsid phosphorylation is approximately 40% of the level seen with intracellular capsids, indicating that the capsid is not completely dephosphorylated during virus assembly. Moreover, it is possible that the pool of phosphorylated capsids serves to "destabilize" nucleocapsids following virus entry so that viral genomes can be efficiently released. In contrast, the nucleocapsid of S46A mutant virus is expected to contain mainly nonphosphorylated capsids that have a high affinity for RNA. During virus disassembly, a minimal level of capsid phosphorylation may be required to maintain the nucleocapsid in a metastable conformation that accommodates the release of the virus genome during virus entry. The strong capsid-RNA interaction within the nucleocapsids of S46A mutant virions appears to inhibit nucleocapsid disassembly, resulting in delayed synthesis of both viral RNA and proteins. Accordingly, it is possible that a critical balance of phosphorylated and nonphosphorylated capsid proteins within the nucleocapsid is essential to maintain its integrity without compromising the efficiency of virus uncoating.

Barring a technical reason, such as low sensitivity of our

methods, failure to detect capsid rephosphorylation during virus entry may reflect the possibility that this process occurs at an earlier stage. For example, in other viruses, such as hepatitis B virus and human immunodeficiency virus, host-encoded protein kinases are packaged into virions (3, 4, 10). In the latter case, the activity of the virion-associated kinase is required for human immunodeficiency virus infectivity (4, 14). Similarly, the failure to detect rephosphorylation of RV capsid after virus entry may indicate that a virion-associated kinase catalyzes phosphorylation of the capsid after virus budding. This is clearly speculative, and it is currently unknown whether any kinases are associated with RV virions. However, a study conducted more than 30 years ago reported that low-level kinase activity copurifies with the nucleocapsids of alphaviruses (33). Unfortunately, the nature of this kinase has not been further investigated. Nevertheless, this report is consistent with the notion that host cell-encoded kinases within togavirus virions mediate timely rephosphorylation of capsids in order to facilitate the release of genomic RNA. Identifying the kinases and phosphatases that are responsible for regulating capsid phosphorylation is critical for understanding where and when these processes occur during replication. Proteomic approaches may afford the best opportunities to identify host-associated protein(s) within rubella virions.

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